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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)* Development of prevention strategies to diminish prostate cancer (PCa) risk is in order. One possible etiologic factor in the development of PCa is cellular exposure to chronic oxidative stress (COS). COS can lead to the accumulation of promutagenic oxidized DNA bases such as 8-hydroxydeoxyguanosine (8-OHdG). The detoxifying enzyme *GSTP1* is inactivated in nearly 100% of PCa. We have successfully developed a model system to determine the role of GSTP1 protein in the response of the human PCa to COS. Through this grant, we have also developed an accurate HPLC-MS-MS method for measuring intracellular reduced and oxidized glutathione. Experiments revealed re-expression of GSTP1 results in an increase in oxidized glutathione following low dose radiation (LDR) but does not significantly diminish intracellular reduced glutathione levels. These data solidify our preliminary results implicating *GSTP1* as a major regulator of oxidative DNA damage and suggest that its inactivation may provide a necessary step in the neoplastic process in PCa. We have also developed an HPLC-MS-MS method for measurement of 8OHdG in genomic DNA. This technique is more quantitative than the older HPLC-ECD method. This new technique has allowed precise measurement of 8-OHdG in DNA extracted from human PCa (LNCaP) xenografts before and after exposure to oxidative injury by LDR. These data reveal that human PCA xenografts expressing GSTP1 do not accumulate significant levels of 8-OHdG following exposure to LDR. These data confirm our ability to measure 8-OHdG via HPLC-MS-MS in DNA extracted from tissues. Finally, we completed a clinical trial evaluating the level of 8-OHdG in PCa and associated normal tissue derived from patients undergoing prostatectomy. These data reveal significant levels of 8-OHdG in the prostate but this was not ubiquitous among all patients evaluated. Together, these data continue to provide support for the argument that *GSTP1* inactivation and subsequent exposure of prostate cells to COS may be part of the neoplastic process.

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Introduction

Reactive oxygen species (ROS) inflict damage on many cellular components, including genomic DNA. Genome lesions accompanying ROS exposure include oxidized bases (1) and have been proposed to be promutagenic in certain systems (2, 3). We hypothesize that prostate epithelial cells with common genetic alterations are provided a significant survival advantage by these alterations when faced with an otherwise toxic oxidative stress. Inactivation of the pi-class glutathione S-transferase gene, *GSTP1*, most commonly occurring by promoter hypermethylation, appears to uniformly accompany human prostatic carcinogenesis (4) and most prostatic intraepithelial neoplasia (PIN) lesions (5). Glutathione S-transferases have been postulated to participate in the defense of normal cells against a variety of carcinogens by catalyzing conjugation reactions between reduced glutathione and reactive electrophiles and oxidants (6). This leads to the possibility that a deficiency in inducible GSTP1 enzyme activity in prostatic epithelial cells might substantially limit their electrophile and oxidant defense capabilities. Genetic alterations that do result as a consequence of oxidative damage are particularly important as they prevent the cell from activating cell death pathways in the face of DNA damage thereby allowing the cell to accumulate potentially promutagenic oxidized DNA adducts like 8-OHdG. Therefore, we reasoned that PCa cells devoid of GSTP1 might be tolerant to chronic oxidative stress and accumulate promutagenic DNA adducts, thereby increasing the risk of neoplastic transformation. Fortunately, there are a number of pharmacologic agents that have anti-oxidant properties and a number of others which specifically target enzymes known to be involved in the production of ROS. There is recent data suggesting several of these agents, including vitamin E and sulindac, may be protective against prostate and other epithelial cancers (7) (8). In fact, there is *in vitro* data suggesting that human PCa cell line growth is also inhibited by sulindac treatment (9). There is conflicting data on whether consumption of any of these agents can produce a decrement in the type and number of promutagenic DNA adducts (10, 11).

The hypotheses of this study are that **(a)** accumulation of oxidized DNA adducts following the chronic oxidant stress of LDR of human prostate cancer cells grown *in vitro* and *in vivo* is a result of *GSTP1* gene inactivation, **(b)** prostatic cells from patients with PCa will possess elevated amounts of oxidized DNA adducts as a result of the chronic oxidant stresses to which they have been exposed, and **(c)** the accumulation of these potentially harmful oxidative DNA adducts can be modulated by the treatment of patients with particular pharmacologic agents. In order to determine if our hypotheses are correct, the following studies are underway: (1) Expand upon and evaluate assays of oxidative DNA damage in the *in vitro/in vivo* LNCaP and LNCaP GSTP1 subclone human PCa model we have developed. (2) Determine the amount of 8-OHdG and other markers of oxidative damage in the DNA of prostatic tissue samples from patients undergoing radical retropubic prostatectomy (RRP) for adenocarcinoma of the prostate. (3) Assay for modulation of 8-OHdG levels in the DNA of prostatic tissue samples from patients following treatment with new and developing pharmacologic prevention/anti-oxidant strategies. Together, these experiments will provide some of the first data set on the role of oxidative DNA damage and its modulation in human prostate cancer and assist in the development of rational PCa chemoprevention strategies.

Body

Task 1: Determine the amount of oxidized DNA base accumulation in our model system of human PCa cells with defective GSTP1 genes as well as those engineered to express GSTP1 polypeptide with enzyme activity following chronic oxidative stress delivered by protracted, low dose irradiation in vitro. Male nude mice will be injected with LNCaP and LNCaP-sublines.

We have completed our *in vitro* analysis of oxidative damage-induced cell death and oxidized DNA base damage accumulation in the human prostate cancer cell line LNCaP, the neo-control line and the three LNCaP-GSTP1 expressing sublines. These data reveal that when compared to any of the GSTP1-expressing

sublines, LNCaP and the LNCaP-neo control cell line, both possessing inactivated *GSTP1* alleles, exhibit significantly greater survival following exposure to the cytotoxic effects of oxidative induced injury inflicted by protracted, low dose radiation (**Figure 1A**) as well as treatment with arsenic trioxide (**Figure 1B**).

Figure 1A

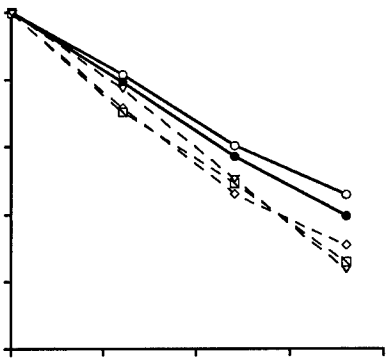
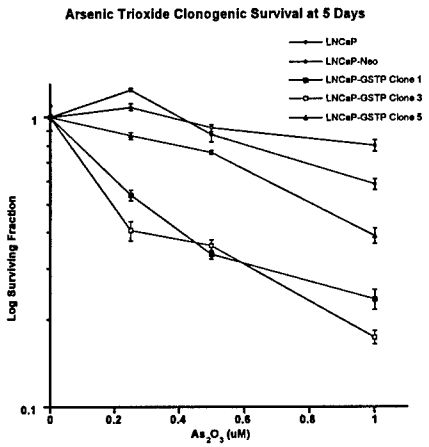


Figure 1B



Our previous work using G.C.-mass spectrometry revealed a significant accumulation of oxidized DNA base adducts, including 8-OHd-guanine, following radiation exposure in the LNCaP neo-control line when compared to the LNCaP-GST-expressing lines. Many investigators believe that HPLC-mass spectrometry (electrospray) (LC/MS-MS) provides a more accurate determination of oxidized DNA base adduct levels and one less likely to produce artifactual oxidation of the DNA during processing. As quantitation of 8-OHd-guanine in DNA samples is critical to the aims of this project, LC/MS-MS has an apparent advantage, particularly when measuring levels from human tissue, where basal levels of oxidized DNA adducts are thought to be low. To this end, we have acquired a new LC/MS-MS (P.E. Biosystems), developed new techniques for the measurement of oxidized base adducts by LC/MS-MS from biologic samples and have shown our ability to measure 8-OHd-guanine at femtomolar (fmol) concentrations. These experiments confirm that LC/MS-MS can accurately and reproducibly measure 8-OHd-guanine in our hands and also suggest that LC/MS-MS is better able to detect low levels (fmol) of oxidized DNA base adducts, which is superior to our previous techniques, (see **Appendices** for example). Moreover, these data now allow us to confidently apply LC/MS-MS in the determination of oxidized DNA base adduct accumulation in prostatic tissue derived from our clinical trial which seeks to determine the ability of candidate chemopreventive agents to modulate these potentially promutagenic adducts.

Finally, we sought to rigorously determine if re-expression of GSTP1 in LNCaP cells would result in global reduction in reduced glutathione (GSH) following exposure to oxidative injury inflicted by LDR thereby resulting an increase in cell death. As GSTP1 conjugates reduced GSH with electrophiles as means of detoxification, it would be theoretically possible that chronic oxidative stress, like that inflicted by protracted LDR, could bankrupt the cells stores GSH rendering the cell extraordinarly vulnerable to continued oxidative injury and increasing cell death. In order to study this, we developed a HPLC-MS-MS method for measurement of intracellular GSH and oxidized glutathione (GSSG). We exposed LNCaP-neo control cells and LNCaP-GSTP1 subclones to LDR at 0.25 Gy/hr for 72 hours and then measured intracellular GSH and GSSG, 6 hours following exposure. Comparison with levels derived from unirradiated cells plated simultaneously was made and served as controls. As seen in **Figure 2A**, there was no reduction of GSH following 72 hr LDR. There was a significant increase in GSSG, as expected, in irradiated GSTP1-expressing cells, but not in irradiated LNCaP-neo control cells (**Figure 2B**).

Figure 2A

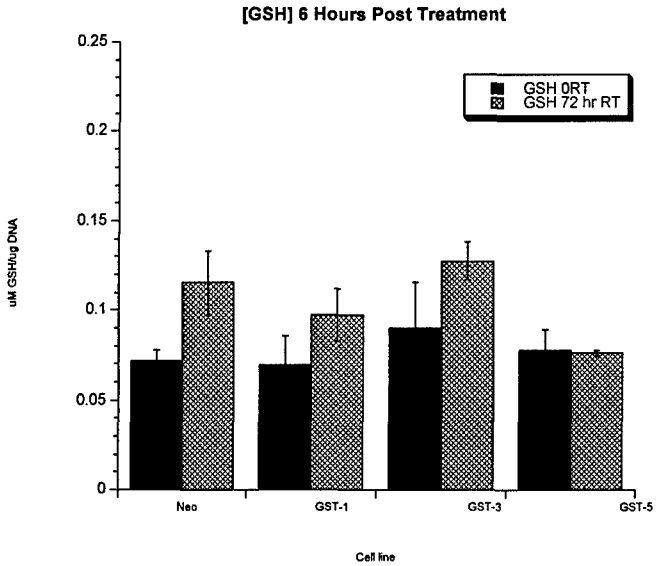
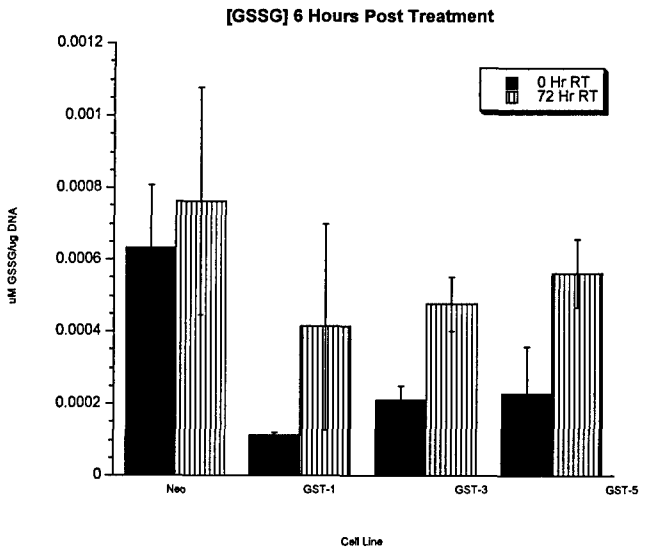


Figure 2B

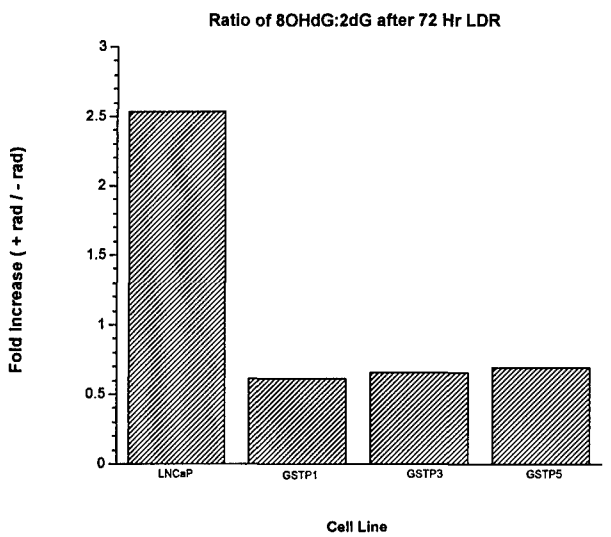


These data eliminate the possibility that the increase in cell death associated with expression of GSTP1 in LNCaP cells is not a result of reduction in levels of the detoxifying protein GSH.

Task 2: Ascertain levels of oxidized DNA base accumulation in LNCaP and GSTP1-subclone xenografts grown in male nude mice. Simultaneously perform immunohistochemical staining for PCNA and p27 polypeptides. Correlation of these data with those from the high performance liquid chromatography-electrochemical detection (HPLC-ECD) and gas chromatography/mass spectrometry with selected ion monitoring (GC/MS-SIM) analyses will be performed in an attempt to determine if PCNA and/or p27 may serve as potential "markers" of oxidative stress. IRB approval for Aim #2 studies will be obtained.

As detailed in **Task 1**, a new method for measuring oxidized DNA base adducts, LC/MS-MS, was developed and equipment was acquired. To determine levels of oxidized DNA adducts in tissue, LNCaP and LNCaP-GSTP1 subclone xenografts were established in nude mice and exposed to whole body LDR (0.25 Gy/hr for 72 hours). Xenografts grown in unirradiated animals served as controls. Fold increases in 8-OHdG/10^6dG were determined (**Figure 3**) and reveal that LNCaP prostate cancer cells, which do not express GSTP1, accumulate significant amounts of 8-OHdG following LDR compared to the LNCaP-GSTP1-expressing subclones.

Figure 3



These data suggest that prostate cancer cells which lack of expression of GSTP1, as found in nearly 100% of human prostate cancers and more than 70% of the pre-neoplastic PIN lesions, seem to be vulnerable to protracted oxidative insults, resulting in accumulation of promutagenic oxidative DNA damage. These *in vivo* data are consistent with our data derived in vitro and continue to argue that prevention strategies seeking to diminish oxidative stress are reasonable.

Oxidative DNA injury typically results in cell cycle arrest in cells with wild type p53 expression. Nearly all prostate cancer cells express wild type p53 protein. We hypothesized that prostate cancer cells experiencing significant oxidative injury would show alteration in markers of replication and cell cycle, p27 and Ki-67. If true, we believe it might be possible to use such markers as an alternative to more complicated assays that actually measure accumulated oxidative DNA adducts, like LC/MS-MS. To study this, we exposed our

prostate cancer model xenografts to the oxidative stress of LDR, the xenografts were harvested, fixed in formalin and embedded in paraffin. Thin sections were created and stained with monoclonal antibodies against p27 and Ki-67. These studies revealed that LNCaP and LNCaP-GSTP1 expressing xenografts exposed to the oxidative stress of total body LDR exhibit a reduction in Ki-67 staining when compared to unirradiated tumors, suggesting a reduction in cellular proliferation (**Figure 4**). LNCaP xenografts showed the largest reduction in Ki-67 staining following irradiation, 10.6 fold, as compared to the LNCaP-GSTP1 expressing xenografts that exhibited reductions in staining between 2.3 and 3.4 fold.

Our original hypothesis sought to determine if enhanced proliferation in the context of continuous oxidative injury would occur preferentially in the non-GSTP1 expressing LNCaP cells. If so, these cells, which preferentially accumulate promutagenic oxidative DNA injury, would be even more likely to serve as source of cancer genesis.

Task 3: Identify and consent patients with adenocarcinoma of the prostate on whom a radical retropubic prostatectomy will be performed. Collect and prepare prostate tissue specimens and perform HPLC-ECD and GC/MS-SIM analyses. Tissue will also be prepared for immunohistochemistry. IRB approval will be obtained for Aim #3 clinical trial.

The protocol for the study of oxidized DNA base damage and immunohistochemical analysis of other potential markers of oxidative stress in radical prostatectomy specimens was written at the time of this grant's submission and included for review. A very protracted process of final protocol approval was undertaken, as documented in the year two report, taking nearly two years to complete. The following summarizes those activities respect to this process.

1) The trial was initially submitted to our JCCI on August 16th 2000 - Both the scientific review committee and the JCCI had comments and questions - which required revisions to the consent and to the protocol - The trial was not approved by the JCCI until **2/13/2001**.
2) **Amendment #1**: Protocol revisions were made in order to modify eligibility criteria and the consent for clarity purposes. (Amendment 1 - submitted 5/14/01- and was approved by the JCCI on 6/05/01)
3) **Amendment #2**: We were notified in the spring of 2001 that the DOD's IRB must also approve the trial before enrollment can began. The DOD had multiple issues that required revisions to the protocol revisions and Consent form - We revised the protocol and the Consent form to reflect the DOD's concerns - We were given conditional approval by the DOD's IRB on 07/30/01. (The committee raised further concerns about the trial and stated that once revisions were made, full approval would be granted.) - We complied. However, due to the OHRP moratorium on research at Johns Hopkins, submission of changes the Johns Hopkins IRB could not occur until 10/26/2001. We did not hear back from the JCCI until 12/10/01 at which time they had questions/and concerns about Amendment #2 - We complied with the requested amendments to the trial (Amendment #3).
4) **Amendment #3**: Amendment #3 was submitted to the JCCI on 12/17/01 - The committee approved **amendments (#2and #3) on 01/28/02**, and informed us that we could begin enrollment.
5) **Amendment #4:** We submitted all of the changes that the Johns Hopkins IRB requested to the DOD IRB. In the interim, the DOD IRB changed several policies concerning research. Hence, amendment #4 had to be created. Amendment #4 was submitted to the JCCI on 2/18/02 and was approved by the JCCI on 2/25/02.
6) The DOD was sent a copy of revision #4, which was approved. However, the DOD instructed us that there were several contractual issues yet to be resolved with Johns Hopkins. These were not

resolved until 04/26/02. It was at that time full approval was granted by both the DOD and the Johns Hopkins JCCI.

Both normal and cancerous prostate tissue was obtained from the prostates of patients undergoing radical retropubic prostatectomy as therapy for clinically-localized adenocarcinoma of the prostate. The median pre-treatment serum PSA for the group was 6.5 ng/ml (range = 1.2-25.1) and the median Gleason score was 6 (range = 6-8). Normal tissue was defined as that tissue with at least 90% normal cells (*i.e.* less than 10% cancerous cells) and cancerous tissue was defined as tissue where 10% or more of the tissue was identified as cancer.

The mean and median fmol 8-OHdG/nmol 2dG ratio for normal tissue was 41.7 and 18.0 respectively. The mean and median fmol 8-OHdG/nmol 2dG ratio for tumor was 64.5 and 13.4 respectively. Neither the mean nor the median 8OHdG levels in normal vs. cancer were statistically different from each other ($p < 0.5$).

The results of this analysis reveal that both normal and cancerous prostate tissues possess variable levels of the oxidized DNA base, 8-OHdG (in fmol per nmol 2dG). While large differences in 8-OHdG levels from cancer and normal prostate tissues were noted in certain patients, we could not confirm that as a group, higher levels of 8OHdG levels existed in prostate cancer as we have previously shown to be true *in vitro* and in human prostate cancer xenografts.

Task 4: Identify, consent and treat patients with adenocarcinoma of the prostate on whom a radical retropubic prostatectomy will be performed.

See Task 3 for details about protocol difficulties. Given this extremely long approval process, two other clinical trials were initiated during the time required for this study's approval. These other trials seek to collect the identical prostate tissue from patients treated with other drugs. As such, this clinical trial has "third priority". Given this priority ranking and despite our best attempts, including opening the trial at both Johns Hopkins Hospital as well as at the Johns Hopkins Bayview hospital, we have not been able to enroll patients. As such, we will not be able to complete Task 4 as proposed.

Key Research Accomplishments

- Development of a useful *in vitro* model of GSTP1 function in human prostate cancer based on the LNCaP cell line.
- Determination of the role of GSTP1 protein expression in the survival of human prostate cancer cells to the oxidative stress inflicted by low dose radiation as well as by arsenic trioxide. Human prostate cancer cells with inactivated *GSTP1* alleles and no GSTP1 protein expression can survive these oxidative stresses significantly better than the same cells engineered to express high levels of GSTP1 protein.
- Development of assay techniques for the measurement of several oxidized DNA bases by LC/MS-MS from biologic specimens.
- Evaluation of oxidized DNA base adduct accumulation following oxidative stress inflicted by protracted, low dose radiation in human prostate cancer cells with and without GSTP1 protein. These experiments suggest that cells with inactivated *GSTP1* alleles and no GSTP1 protein expression not only survive the oxidative stress of protracted, low dose radiation, but also accumulate significantly greater amounts of the potentially promutagenic oxidized bases, 8-OHd-guanine and 8-OHd-adenine.

- Successful application of newly developed LC/MS-MS technique to measurement of 8-OHdG in DNA extracted from human prostate cancer xenografts.
- *In vivo* confirmation of *in vitro* results revealing that LNCaP subclones expressing GSTP1 do not accumulate significant increases in 8-OHdG levels following exposure to the oxidative stress of LDR.
- Evaluation of proliferative markers following LDR-induced oxidative injury by immunohistochemical analysis. This analysis reveals that LNCaP prostate cancer xenografts expressing GSTP1 exhibit significantly greater reductions in the proliferative marker, Ki-67 following LDR, compared to LNCaP-GSTP1 expressing xenografts.
- First in-depth analysis of 8OHdG levels from normal and cancerous prostate tissue derived from patients with clinically-localized adenocarcinoma of the prostate cancer undergoing radical retropubic prostatectomy as primary therapy. This inaugural analysis reveals large variations in 8OHdG levels in cancer and normal tissue from individual patients but no significant differences between normal and cancerous tissue when evaluating the entire group of patients.

Reportable Outcomes

- Development of relevant human prostate cancer cell lines with and without GSTP1 protein expression.
- Identification of a GSTP1-mediated oxidative damage tolerance phenotype in human prostate cancer cells.
- Development of an accurate, reproducible and highly sensitive LC/MS-MS method for measurement of GSH and GSSG in human prostate cancer cells and other cell types.
- Determination that re-expression of GSTP1 protein in prostate cancer cells lacking GSTP1 expression (LNCaP) does not result in GSH "bankruptcy" following oxidative insults.
- Development of an accurate, reproducible and highly sensitive LC/MS-MS method for measurement of 8-OHdG in DNA from human prostate cancer cells and other cell types.
- Recognition of accumulation of promutagenic oxidized DNA base adducts (8-OHdG) in prostate cancer cells lacking GSTP1 protein (LNCaP) when compared to GSTP1-expressing subclones of LNCaP.
- Measurement of promutagenic oxidized DNA base adduct (8-OHdG) in both normal and cancerous prostate tissue derived from patients with prostate cancer who are undergoing radical prostatectomy.

Conclusions

Taken together, these data suggest that the most common genetic alteration known in human prostate cancer, hypermethylation of the *GSTP1* promoter leading to gene inactivation, can result in: 1) oxidative DNA damage tolerance as reflected by an increased clonogenic survival following protracted low dose radiation and exposure to arsenic trioxide and 2) accumulation of potentially promutagenic oxidized DNA base adducts. We have confirmed that LC/MS-MS is an appropriate and highly sensitive method for determining levels of 8-OHdG in DNA derived from both cells and prostate cancer xenografts. Using this new method, we have shown that the fold increase in 8-OHdG in DNA extracted from irradiated LNCaP cells grown *in vitro* and *in vivo* is significantly greater than levels from the GSTP-1-expressing subclones. We have also shown that the enhanced cell death seen in the GSTP1-expressing subclones of LNCaP following protracted, oxidative injury by LDR is not a result of GSTP1-induced reduction in GSH. Finally, while significant differences in 8-OHdG levels from cancer and normal prostatic tissue exist in individual patients with prostate cancer, these difference are not uniform across all patients tested and do not translate into statistically significant differences. This suggests that

while alterations in GSTP1 exist and are seemingly relevant in the context of prostate cancer initiation and do relate to *in vitro* oxidative DNA damage tolerance, we cannot also state that such alterations are uniformly relevant across patients with actual prostate cancer. We have not tested whether accumulation of 8OHdG levels would be uniformly increased in preneoplastic lesions such as PIN.

Overall, these critical alterations in phenotype based on GSTP1 expression may be some of the earliest and most critical factors in the neoplastic process of human prostate cancer. Further work is necessary to determine if alterations in GSTP1 are more relevant in the context of preneoplastic lesions like PIN or PIA and drive accumulation of substantial promutagenic oxidized bases not seen across a population of patients with prostate cancer. Ultimately, this work and future work by us and others helps increase the foundation for concepts important for the design of rational chemoprevention strategies, including those seeking to minimize cellular oxidative DNA damage.

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